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14. ABSTRACT Mitochondria are the powerplants of the cell. They produce the ATP necessary for the neurons to engage in reactions geared toward their proper function. Mitochondria contain a series of enzymes, in a chain-like array, that pass electrons along this chain via proton motive force which is initiated by complex I, the first of this series of enzymes. Complex I deficiency is considered one of the hallmarks of Parkinson's Disease as it contributes greatly to the energy crisis in the neurons. In an earlier study, bypassing this complex I deficiency using D-β-hydroxybutyrate (DβHB) in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of PD, dopamine neurons in the substantia nigra pars compacta were protected. Our goal in this study is to assess the effects of DβHB analogues to ascertain if they are longer-acting compounds than the parent compound. Although obtaining the first and only drug at the moment was quite difficult (it took close to 10 months, we have now initiated our first experiment which is to determine the effective dose to use in future experiments.					
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Scientific Progress Report (W81XWH-10-1-0539: July 2012)

Pre-Clinical Testing of New Hydroxybutyrate Analogs

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's Disease (AD) (Fahn and Przedborski, 2009). It is characterized clinically by resting tremor, rigidity, akinesia, and postural instability (Fahn and Przedborski, 2009). These motor manifestations are attributed mainly, though not exclusively, to the loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and their dopaminergic terminals in the corpus striatum of the nigrostriatal pathway in the brain. Biochemically, PD presents with a profound loss of DA levels in the striatum, which, in part, accounts for the noted motor manifestations (Hornykiewicz and Kisk, 1987). Although current therapy includes dopaminergic agonists and cholinergic antagonists, the most reliable and most common therapy remains Levodopa (L-DOPA), a precursor for dopamine (DA) (Fahn and Przedborski, 2009).

PD is thought of as a multi-faceted disease (Reichmann and Jost, 2008), in that it is a disease not based on a single cause, but on many interacting causes. Recent evidence suggests that mitochondrial dysfunction may be one contributing factor (Fahn and Przedborski, 2009). Mitochondria are the powerhouses of the cell and, as such, produce the energy necessary for the cell to function. Movement of electrons through the mitochondrial electron transport chain (METC), a series of enzymes (complexes I, II, III, IV, V) starts with proton motive force at the complex I site, the first of the METC series of enzymes. Electrons move down the chain to eventually produce ATP. However, when complex I is compromised, as is reported in PD (Fukae et al, 2007) and demonstrated in the MPTP mouse model of PD (Cassarino et al, 1997), mitochondria become dysfunctional as mitochondrial membrane potential collapses, ATP production is reduced and protons no longer travel properly along the chain. Thus, the neuron experiences energy crisis and respiratory failure, which means that oxidative phosphorylation is compromised and there is an increase in the presence of the superoxide radical.

Part of PD neurochemistry is a deficit in complex I activity in the METC (Suzuki et al, 1997) and this neurochemical deficit can be reproduced using the MPTP mouse model of PD. Previously, we demonstrated that we could overcome the complex I deficit using β -hydroxybutyrate, a ketone body, in the acute MPTP mouse model (Tieu et al, 2003). Infusion of this compound partially protected dopamine neurons in the SNpc against the damaging effects of MPTP and improved the motor deficits elicited by MPTP. This was accomplished by enhancing oxidative phosphorylation through augmentation of complex II activity. The only drawback to the use of β -hydroxybutyrate is that it is short-

acting and has to be delivered via alzet pumps in order to maintain steady-state blood levels. Recently, we have received a new compound, based on the skeleton of β -hydroxybutyrate. This new compound, glyceryl tris(3-hydroxybutyrate) (G3HB) from Eastman Chemical Company, supposedly has a much longer half-life. Not much is known about the new compound and further, its chemical and toxicological properties have yet to be fully tested.

Body of Work

SA I: we will examine the effects of the beta-hydroxybutyrate analogs on mitochondrial function and HDAC activity *ex vivo* and compare the obtained results with those of mitochondrial exposure to D β HB.

SA II: we will assess the neuroprotective qualities of these beta-hydroxybutyrate analogs in an *in vivo* setting in the MPTP mouse model of neurodegeneration. SA II has four parts.

Part I, as we have done previously with D β HB, we will first initiate the infusion, via Alzet mini-osmotic pumps, of the compounds analogues of D β HB with longer half-lives and a suitability for single daily dosage. Twenty-four hours after pump implantation, MPTP will be administered in an acute regimen. Animals will be perfused at 2 and 7 days after MPTP administration and the integrity of the nigrostriatal system will be assessed. Mac-1 and GFAP immunostaining will be done to gauge inflammation as it relates to HDAC activity. Tyrosine hydroxylase and Nissl stained neurons will be counted and complex II histochemistry will be performed.

Part II, we will prepare a new set of implanted mice treated with MPTP and motor function will be assessed on the rotarod. We will then remove the pumps and use regional sections from these brains to measure nigrostriatal levels of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA).

Part III, a third set of implanted animals will be prepared and used for brain and blood measurements of MPTP and MPP+ will be measured at 90 minutes after the last dose of MPTP in our acute regimen to examine MPTP metabolism in the presence of compound A and B. We will also measure D β HB levels in blood and brain at selected times during the infusion of compound A and compound B to demonstrate whether these compounds can act as replacements for D β HB. To test the suitability for single daily dosing,

Part IV will involve single daily dosing starting at twenty-four hours prior to the initiation of MPTP. Single daily dosing will continue for four additional days and mice will be sacrificed 7 days after the last dose of MPTP. Brains will be assessed as in parts I and II.

Key Accomplishments

In our hands, G3HB was first tested in C57bl/6 mice from Charles River, as this is the strain that we have tested from all of the Charles River breeding houses in the United States and found the the mice from Kingston NY gave significant cell death with less than 5% animal death. Thus, we have used these mice in all of our MPTP studies for the past 20 years. In our initial experiment with these mice, acute MPTP (18 mg/kg free base x 4 doses over 8 hours) and the highest concentrations of G3HB (0.8 and 1.6mm/kg/day) using Alzet pumps for the delivery of the G3HB. As noted in A., there was ~a 55% loss of TH+ neurons in the SNc of the C57bl/6 mice (green:saline vs red: MPTP) which was reversed to 70% of control (orange: 0,8mmol/kg/day G3HB + MPTP and to `91% of control (yellow 1.6mmol/kg/day + MPTP). Although there was a small loss in ventral tegmental area (VTA) TH+ neurons, unlike in the SNc, the TH+ neuronal loss in the VTA was not significant.

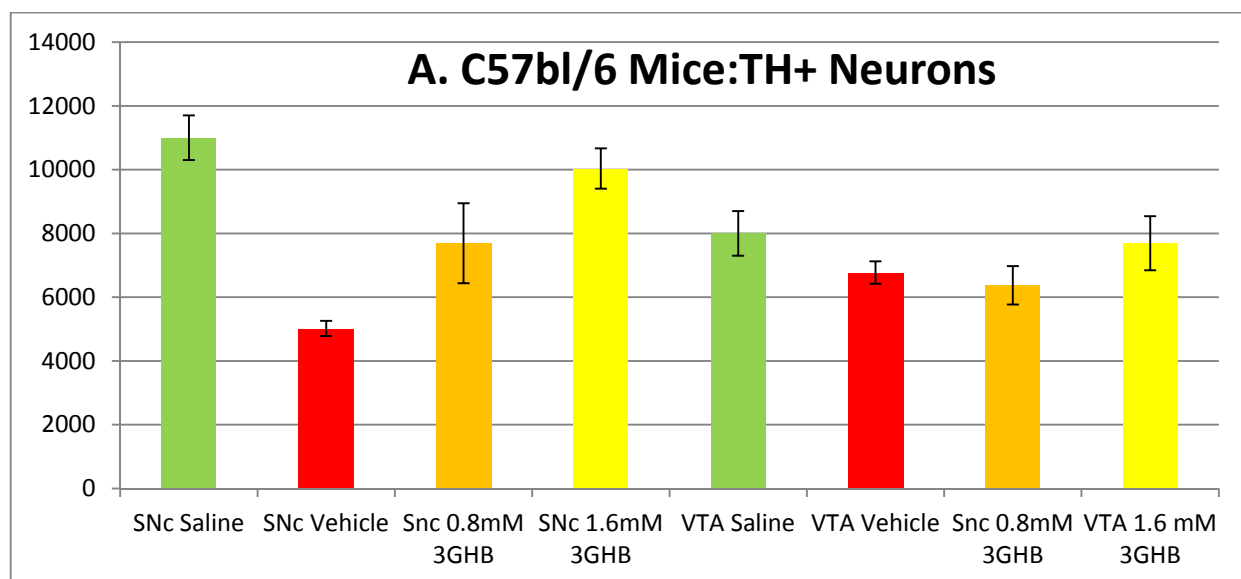


Figure A. TH+ neuron counts for the SNc and the VTA of saline (green), MPTP, 18 mg/kg ip x 4 (red), MPTP + 0.8 mmol/kg/day G3HB) (orange), MPTP + 1.6 mmol/kg/day; N/group range from 3 to 8.

Reportable Outcomes

Although the results with C57bl/6 are good, the problem here is that the ensuing experiments using these mice were not fruitful as all of the MPTP-treated and MPTP-G3HB treated mice died. We tried repeating our first study using 4 different concentrations of the G3HB with MPTP and all of the mice died. Thinking that this was some kind of anomaly, we repeated the study a third time with the same results. We are not sure why all of the animals died, but we have considered gene drift, a mix-up in the

mouse order, mice being shipped from the wrong breeding house. While we are still in the dark about the deaths, since we are examining other strains in another study, we tried two other strains, SJL and C3H that were used in another study. About 50% of the SJL mice died whereas none of the C3H mice died. Figure B shows the TH+ neuron counts for one side of the SNc. In these mice, we saw ~36% TH+ neuron loss which was ~90% corrected with G3HB, 1.6mmol/kg/day. Because of the cell death difference between the C57 and the C3H mice, we have pushed to dosing of MPTP to 20 mg/kg in these C3H mice. If this proves fruitful, we can perform our studies in the C3H mice, but we will investigate the problem with the C57bl/6 mice from Charles River labs.

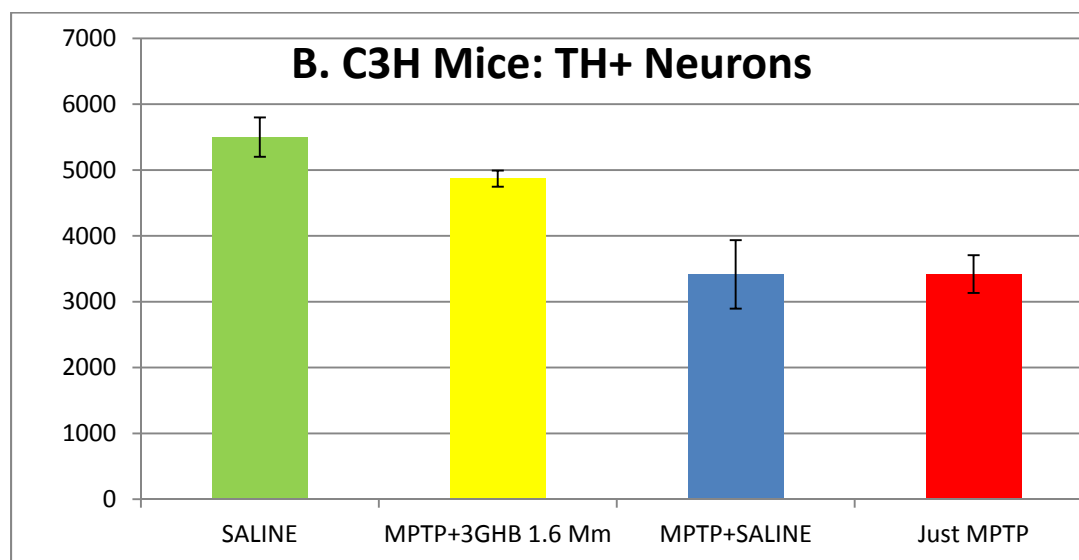


Figure B. TH+ neuron loss in MPTP-G3HB treated mice. Saline, MPTP-G3HB, and MPTP-Saline were pump-implanted. Just MPTP had no pump. N=4/group; no animal died.

Conclusions

From our studies thus far, we know that the provided compound G3HB is neuroprotective toward the TH+ SNpc neurons but that its use may be strain dependent.

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C, Caspersen C, Teismann P, Wu DC, Yan SD, Naini A, Vila M, Jackson-Lewis V, Ramasamy R, Przedborski S. [D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease.](#) J Clin Invest. 2003 Sep;112(6):892-901.

Appendices follow: Figures and Tables

Material Safety Data Sheets for Glycerol tris(3-hydroxybutyrate).

EASTMAN

Eastman Chemical Company
Eastman Research Division
P.O. Box 1972
Kingsport, Tennessee 37662

PURITY PROFILE*

25 March 2011

Product Name: Glycerol tris(3-hydroxybutyrate)

Ship to:

Attn: Dr. Serge Przedborski/Dr. Vernice Jackson-Lewis
Columbia University, MNC
P&S 4-401
630 West 168th Street
New York, NY 10032
Tel: (212)305-8689

Date shipped: 11 April 2011
Containers shipped: 1
Weight shipped: 80 g

Lot Number EX001250-031

Properties

Sample

Method(s)

identity
appearance
wt% assay
GC assay

consistent with structure
pale yellow oil
>98%

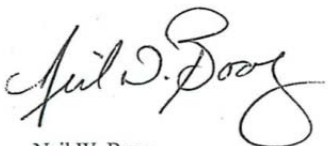
NMR
wt% NMR

glycerol tris(3-hydroxybutyrate)
glycerol bis(3-hydroxybutyrate)
residual solvent (ethyl acetate)

93.7%
2.6%
<0.5%

GC derivatization area%
GC derivatization area%
wt% NMR

MW 350.36



Neil W. Boaz
Eastman Representative
423-229-8105
Email nwboaz@eastman.com

**This product is subject to ongoing development. The results provided in this Purity Profile were obtained by analyzing the Batch/Lot described and may or may not be representative of any past or future Batches/Lots. The methodology and/or techniques of analysis used to obtain these results may or may not be validated. The recipient should independently determine whether this product meets their specifications and is technically suitable for their intended purpose. For additional information regarding this product and its analysis, please contact your Eastman representative. This material is NOT for human consumption.*

4/13/11
JC

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MATERIAL SAFETY DATA SHEET

Revision Date: 04/08/2011
MSDSUSA/ANSI/EN/150000072403/Version 2.0

1. CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Product Name	Glyceryl tris(3-hydroxybutyrate)
Product Identification Number(s)	33046-00, E3304601
Manufacturer/Supplier	Eastman Chemical Company 200 South Wilcox Drive Kingsport, TN 37660-5280 US +14232292000
MSDS Prepared by	Eastman Product Safety and Health
Chemical Name	Not applicable
Synonym(s)	985287
Molecular Formula	Not applicable
Molecular Weight	Not applicable
Product Use	research and development sample
OSHA Status	assumed hazardous; not fully investigated

For emergency health, safety, and environmental information, call 1-423-229-4511 or 1-423-229-2000.

For emergency transportation information, in the United States: call CHEMTREC at 800-424-9300 or call 423-229-2000.

2. COMPOSITION INFORMATION ON INGREDIENTS

(Typical composition is given, and it may vary. A certificate of analysis can be provided, if available.)

Weight %	Component	CAS Registry No.
>90%	Glyceryl tris(3-hydroxybutyrate)	135413-30-8
<5%	Glyceryl bis(3-hydroxybutyrate)	Not assigned
<1%	ethyl acetate	141-78-6

3. HAZARDS IDENTIFICATION

WARNING!
THE PHYSICAL-CHEMICAL AND TOXICOLOGICAL PROPERTIES OF THIS MATERIAL HAVE NOT BEEN FULLY INVESTIGATED

HMIS® Hazard Ratings: Health - 2, Flammability - 1, Chemical Reactivity - 0

HMIS® rating involves data interpretations that may vary from company to company. They are intended only for rapid, general identification of the magnitude of the specific hazard. To deal adequately with the safe handling of this material, all the information contained in this MSDS must be considered.

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MATERIAL SAFETY DATA SHEET

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exposure limits. If exposure limits have not been established, maintain airborne levels to an acceptable level.

Respiratory Protection: If engineering controls do not maintain airborne concentrations below recommended exposure limits (where applicable) or to an acceptable level (in countries where exposure limits have not been established), an approved respirator must be worn. Respirator type: Air-purifying respirator with an appropriate, government approved (where applicable), air-purifying filter, cartridge or canister. Contact health and safety professional or manufacturer for specific information.

Eye Protection: Wear safety glasses with side shields (or goggles).

Skin Protection: Wear chemical-resistant gloves, footwear, and protective clothing appropriate for the risk of exposure. Contact health and safety professional or manufacturer for specific information.

Recommended Decontamination Facilities: Eye bath., Washing facilities., Safety shower.

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical Form: Viscous Liquid

Color: Yellow

Odor: Slight

Specific Gravity: < 1

Boiling Point: 200 °C 0.5 mm Hg

Solubility in Water: Appreciable

Flash Point: > 93 °C (estimated)

Thermal Decomposition Temperature: Thermal stability not tested. Low stability hazard expected at normal operating temperatures.

10. STABILITY AND REACTIVITY

Stability:

Not fully evaluated. Materials containing similar structural groups are normally stable.

Incompatibility:

Material reacts with Strong oxidizing agents..

Hazardous Polymerization:

Will not occur.

11. TOXICOLOGICAL INFORMATION

Acute toxicity data, if available, are listed below. Additional toxicity data may be available on request.

12. ECOLOGICAL INFORMATION

Acute toxicity data, if available, are listed below. Additional toxicity data may be available on request.

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This material has not been tested for environmental effects.

13. DISPOSAL CONSIDERATIONS

Dispose of waste and residues in accordance with local authority requirements. Incinerate. Since emptied containers retain product residue, follow label warnings even after container is emptied.

14. TRANSPORT INFORMATION

Important Note: Shipping descriptions may vary based on mode of transport, quantities, package size, and/or origin and destination. Consult your company's Hazardous Materials/Dangerous Goods expert for information specific to your situation.

DOT (USA)

Class not regulated

Sea - IMDG (International Maritime Dangerous Goods)

Class not regulated

Air - ICAO (International Civil Aviation Organization)

Class not regulated

15. REGULATORY INFORMATION

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This product has been classified in accordance with hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

WHMIS (Canada) Status: controlled

WHMIS (Canada) Hazard Classification: D/2/B

SARA 311-312 Hazard Classification(s):
immediate (acute) health hazard

SARA 313:

Carcinogenicity Classification (components present at 0.1% or more): none, unless listed below

TSCA (US Toxic Substances Control Act): One or more components of this product are not listed on the TSCA inventory. In the USA, commercial industrial use is restricted to FDA-regulated applications.

16. OTHER INFORMATION

Visit our website at www.EASTMAN.com or email emnmsds@eastman.com

The information contained herein is based on current knowledge and experience; no responsibility is accepted that the information is sufficient or correct in all cases. Users should consider these data only as a supplement to other information. Users should make independent determinations of suitability and completeness of information from all sources to assure proper use and disposal of these materials, the safety and health of employees and customers, and the protection of the environment.

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